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Research Article

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Methylation of hemoglobin to enhance flocculant performance

Matthew Essandoh,[a*] Rafael A Garcia[a] and Gary D Strahan[b]

Abstract

BACKGROUND: Synthetic organic polymer flocculants can be highly effective at clarifying suspensions, but these substances may also have negative environmental and health effects. Relatively few studies have been done using biobased flocculants, which are more environment friendly. Hemoglobin (Hb) has previously been demonstrated to be a promising flocculant of kaolin and lignin suspensions. This study examines the methylation of Hb side chain carboxyl groups for the purpose of improving its flocculation performance at near-neutral pH.

RESULTS: Potentiometric titration of methylated Hb (MeHb) showed an approximately 28% degree of methylation when the Hb was suspended in methanol with 0.8 mol L^{-1} HCl for 48 h. Under some conditions, MeHb clarified suspensions of kaolin at one-quarter the dose that was required for Hb. Furthermore, MeHb exhibited flocculant activity over a wide pH range, compared with Hb. The percentage of original turbidity removed was 37% for Hb while 60% of the original turbidity was removed for MeHb at near-neutral pH (pH = 6.8).

CONCLUSIONS: Very small doses of methylated hemoglobin (MeHb) rapidly clarify suspensions of kaolin. The potential of MeHb as a biobased flocculant for the clarification of water for industrial or municipal use was demonstrated.

Keywords: hemoglobin; bioflocculant; methylation; water treatment; kaolin

INTRODUCTION

Flocculants are substances which aid water clarification by causing suspended particles to aggregate and later settle. They are used in several industrial fields including wastewater treatment, mineral extraction, agricultural irrigation, and land management. Polyacrylamide (PAM) and its derivatives are among the most widely used synthetic polymer flocculants. However, PAM's monomer (acrylamide) and other degradation products are highly mutagenic and carcinogenic, and sometimes lead to low microbial activity in activated sludge.[1,2] Also, the use of PAM-based flocculants can lead to the build-up of acrylamide concentrations in sludge basins.[3] Environmental persistence of synthetic polymers is well known. It is therefore not surprising that various concerns have been raised across the globe against the use of synthetic organic polymeric flocculants (SOPF), especially PAM.[4] Biobased flocculants may be effective substitutes for PAM.

Annually, approximately 2 million tons of animal blood are produced in the US as a by-product from slaughterhouses.[5] Blood has a high concentration of the protein hemoglobin (Hb) and Hb is easily isolated from whole blood. Hb has been shown to be a very effective flocculant of kaolin and lignin suspensions, at relatively low doses.[6] A major drawback of Hb-flocculant is that its performance drops off rapidly as the pH increases above 5.5. Expanding the pH window beyond pH 5.5 would enhance the utility of Hb as a bio-based substitute for PAM.

Although methylation of proteins has been reported often in the literature, relatively little research has been performed on methylated proteins as flocculants. The flocculation of diatomite by methylated egg albumin and soy protein have been reported.[7,8]

Clay particles (kaolin) have a negative charge and are made primarily of silicate. The negative charges cause the clay particles to repel each other, preventing them from aggregating. Proteins, however, have both positive and negative charged groups exposed to the solvent. Reducing the amount of negatively charged groups on a protein may increase the attraction between the protein and negatively charged particles. Methylation of the hemoglobin to eliminate the carboxylic acid groups will increase the basicity and the net positive charges on the surface of the hemoglobin which can lead to enhanced flocculation performance. This research focuses on methylation of Hb and its use as a renewable flocculant. The synthesized methylated hemoglobin

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(MeHb) is characterized systematically to provide an in-depth knowledge of the changes occurring after methylation, and is tested for its ability to promote the flocculation of clay at different pH values, settling times, and flocculant doses.

EXPERIMENTAL

Materials

Kaolin with the trade name 'Polygloss 90' from Huber Engineered Materials (Atlanta, GA, USA) was a gift from the MF Cachat Company (Lakewood, OH, USA). All other reagents and materials used in this study, including hemoglobin, were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. The hemoglobin used in this study was prepared from washed, lysed and dialyzed erythrocytes. Detailed descriptions of the preparation and processing of the Hb can be found on the manufacturers' website.

Methylation procedure

Methylation was carried out according to the method reported by Fraenkel-Conrat and Olcott (1945) with some modifications.[9] In summary, 3% (w/v) lyophilized bovine hemoglobin (Hb) was suspended in methanol. Hydrochloric acid was added to the reaction medium so that the final concentration was 0.8 mol L^{-1}. The sample was shaken continuously for 48 h at room temperature. The methylated bovine hemoglobin (MeHb) was recovered by centrifugation at 10 000 × *g* for 15 min. MeHb was then lyophilized and stored until needed.

Degree of methylation analysis

The degree of methylation was estimated following the protocol of Seki and Suzuki with modifications.[7] Potentiometric titration was carried out using an autotitrator (Model 842, Metrohm Ltd, Herisau, Switzerland). The autotitrator was programmed to add the titrant until pH 11 (stop criterion) was achieved. In brief, 0.2 g of the Hb or MeHb were dissolved in 0.2 L of water, and then stirred continuously. The ionic strength of the solution was adjusted to 0.1 mol L^{-1} using NaCl, before purging the sample under nitrogen environment for 30 min. The titration was then performed (from pH 3 to 11) using 0.1 mol L^{-1} NaOH under nitrogen environment. The second derivative of the pH values vs volume (mL) of NaOH used was plotted and the equivalence point was taken as the volume at which the second derivative was zero. The total carboxylic acid groups before and after methylation was used to estimate the extent of methylation using the formula below:

$$TCAG = \frac{N \cdot V\,(45/1000)}{M} \quad (1)$$

where $TCAG$ = total carboxylic acid group content (mol g^{-1}); N = concentration of the titrant (mol L^{-1}); V = volume at the equivalence point (L); and M = mass of oven dried sample (g).

Quantification of hemoglobin in Hb and MeHb

The amount of hemoglobin was quantified in Hb and MeHb samples using the alkaline heamatin D-575 method with some slight modification.[10] Briefly, alkaline haematin detergent 'AHD' reagent was prepared by adding 25 g of Triton-X 100 to 0.1 mol L^{-1} of 1 L NaOH. 20 μL of a previously centrifuged Hb or MeHb solution was added to a test tube containing 3 mL of the 'AHD reagent'. The mixture was then vortexed and allowed to stand for about 5 min at room temperature. The absorbance at 575 nm was taken and the concentration was determined from a calibration graph prepared using a chlorohematin standard.

Fourier transform infrared spectroscopy (FTIR) analysis

The infrared spectra for both hemoglobin and methylated hemoglobin were obtained using a Thermo Nicolet 6700 FT-IR (Thermo Electron Corporation, Madison, WI, USA) spectrometer with DTGS detector. A total of 64 scans were taken from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}.

Circular dichroism analysis

A circular dichroism (CD) spectrometer (model 420, Biomedical Inc., Lakewood, NJ, USA) equipped with a photomultiplier tube was used to study the Hb and MeHb secondary structure content. The CD spectra were taken using a sample concentration of 3 µmol L^{-1} at 25°C. The spectra were recorded with a bandwidth of 1 nm and an averaging time of 5 s. A blank was also prepared for baseline correction. Samples were centrifuged at 5000 × *g* for 5 min prior to analysis. A high transparency, 1 mm in path length quartz cell was used for the measurement. The CD spectra were recorded within the far UV region (190–250 nm) and the results are shown in molar ellipticity as a function of wavelength. All spectra were normalized by subtracting the baseline obtained for the blank.

Nuclear magnetic resonance (NMR) procedure

All spectra were acquired at 25°C in D_2O, with the sodium salt of 3-(trimethylsilyl)-propionic acid-d_4 (TSP) added for signal referencing. Spectra of the MeHb were also acquired with the addition of 0.9% w/v NaCl to enhance its solubility and a comparison spectrum was acquired under the same conditions for the non-methylated Hb. Presaturation ^{1}H (proton) spectra of both samples were acquired using sweep-widths of 5205 at 400 MHz and 9615 Hz at 600 MHz, using 32 k data points, and were acquired with a 45° pulse angle with a 30 s relaxation delay and a pre-saturation pulse of 1.5 s. Solution-state NMR spectra were recorded at 14 Tesla on a VNMRS NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) using a 5 mm One NMR probe with z-axis pulsed field gradients. As the MeHb is flocculated in solution, adequate ^{1}H resolution could not be achieved by standard solution-state NMR. Instead, the pre-saturation ^{1}H spectrum was recorded at 9.4 Tesla on a Varian Inova NMR spectrometer (Palo Alto, CA, USA) using a 40 μL MAS (magic-angle spinning) nanoprobe equipped z-axis pulsed field gradients. The resulting spectrum still suffers from significant line-broadening, but it is significantly improved over the standard solution state spectrum. The ^{13}C spectra of both samples were acquired in solution at 150 MHz, with a sweep-width of 37 878 Hz and 64 k data points, and were signal averaged over 50 000–100 000 transients, utilizing a 45° pulse angle and a 1 s relaxation delay. The ^{13}C 135-DEPT (distortionless enhancement polarization transfer) experiments were acquired using the same parameters as the ^{13}C spectra, with the C–H coupling constant set to 135 Hz. The ^{13}C and ^{13}C-DEPT spectra of the methylated sample have slightly broader linewidths, but are still interpretable due to the greater signal dispersion of the ^{13}C nucleus. Attempts to acquire HSQC or HMQC spectra of the methylated sample were unsuccessful due to the fast relaxation of the signal. All data were processed and baseline corrected using Agilent's VNMRJ 4.2 software.

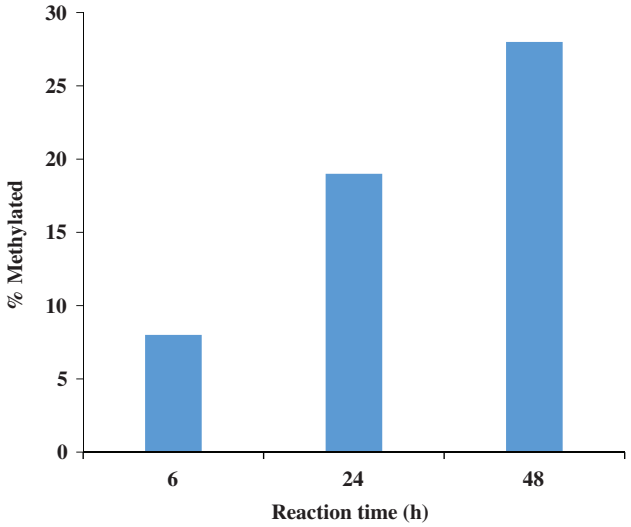

Figure 1. Percentage methylated when 3% (w/v) lyophilized bovine hemoglobin (Hb) was suspended in methanol containing 0.8 hydrochloric acid at room temperature.

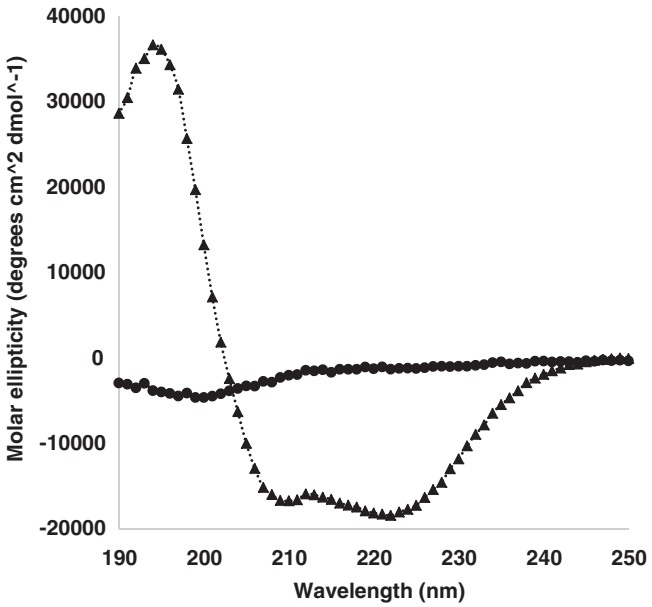

Figure 2. Circular dichroism spectra of Hb (▲) and MeHb (●) in aqueous solution in the far UV region. Experimental conditions: temperature 25°C, sample concentration 3 μmol L^{-1}, bandwidth 1 nm, averaging time 5 s.

Flocculation test with kaolin

Flocculation experiments were carried out using a method which was designed in the author's lab for evaluation of experimental flocculants.[11] Kaolin suspension of 3 g L^{-1} in 25 mmol L^{-1} Malic-MES-Tris buffer (MMT) at different pH (4.5 to 8.5) containing 0.01% thimerosal was prepared. Microorganisms may grow in intermediate pH buffers. Therefore, kaolin suspension can be stored in the refrigerator or 0.01% thimerosal can be added to prevent the growth of microorganisms. It is worth noting that the addition of 0.01% of thimerosal does not change the stability of the suspension. The initial turbidity of a 24 mL kaolin suspension was measured in a cylindrical glass vial with plastic caps. An appropriate aliquot of the flocculant was added and the suspension and flocculant were shaken at 400 rpm for 1 min followed by 200 rpm

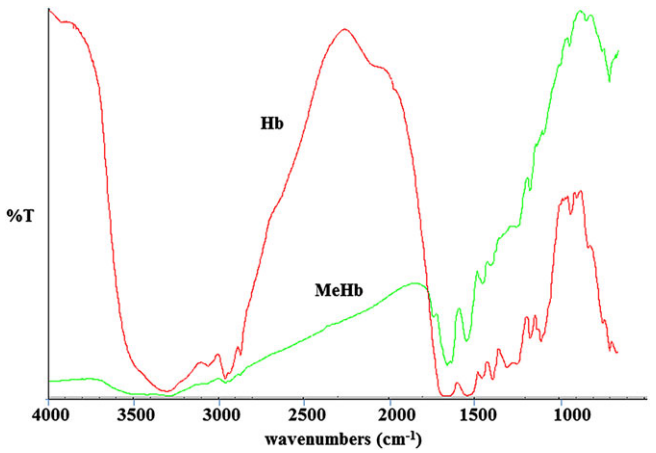

Figure 3. FTIR of Hb and MeHb. A total of 64 scans were taken from 4000 to 600 cm^{-1} with a resolution of 4 cm^{-1}.

for 15 min. A control was also prepared in similar manner without the addition of the flocculant. The glass vials were placed in a tube rack and left undisturbed at 20 ± 1°C for 6 h before measuring the final turbidity. Previously, we have found that the turbidity values are fairly constant after 6 h. Therefore measurements were taken at 6 h. A minimum of four replicates were prepared. Kaolin clarification effectiveness (KCE) was computed using:

$$KCE_{(Conditions)} = \log_{10}\left(\frac{T_i}{T_f}\right) \tag{2}$$

where T_i and T_f are the initial and final turbidity, respectively, and the subscript (conditions) represent the standard conditions or the conditions used by the experimentalist. Standard deviation of KCE, S_{KCE}, was calculated using:

$$S_{KCE} = \left(\frac{0.434}{\sqrt{n}}\right)\sqrt{\frac{s_f^2}{T_f^2} + \frac{s_i^2}{T_i^2}} \tag{3}$$

where s_i and s_f are the standard deviations of the initial and final measurements, respectively, and n is the number of replicates.

RESULTS AND DISCUSSION

Degree of methylation

When bovine hemoglobin (Hb) was esterified with methanol at different reaction times, different degrees of methylation were obtained. It has been reported that methylation is a specific reaction that affects only the carboxylic acid groups on proteins while other functional groups like indole, amino, phenolic, and thiol groups remain the same.[9] The degree of methylation was quantified by potentiometric titration by comparing the total carboxylic acid groups before and after methylation. Figure 1 shows reaction times of 6, 24, and 48 h yielding 8%, 19%, and ~28% degree methylation, respectively. All subsequent studies were therefore done using the MeHb that was ~28% methylated. This is comparable with prior work in which the extent of esterification of β-lactoglobulin was 14%, 23%, 28%, and 32% for reaction times of 16, 24, 48, and 72 h, respectively.[12]

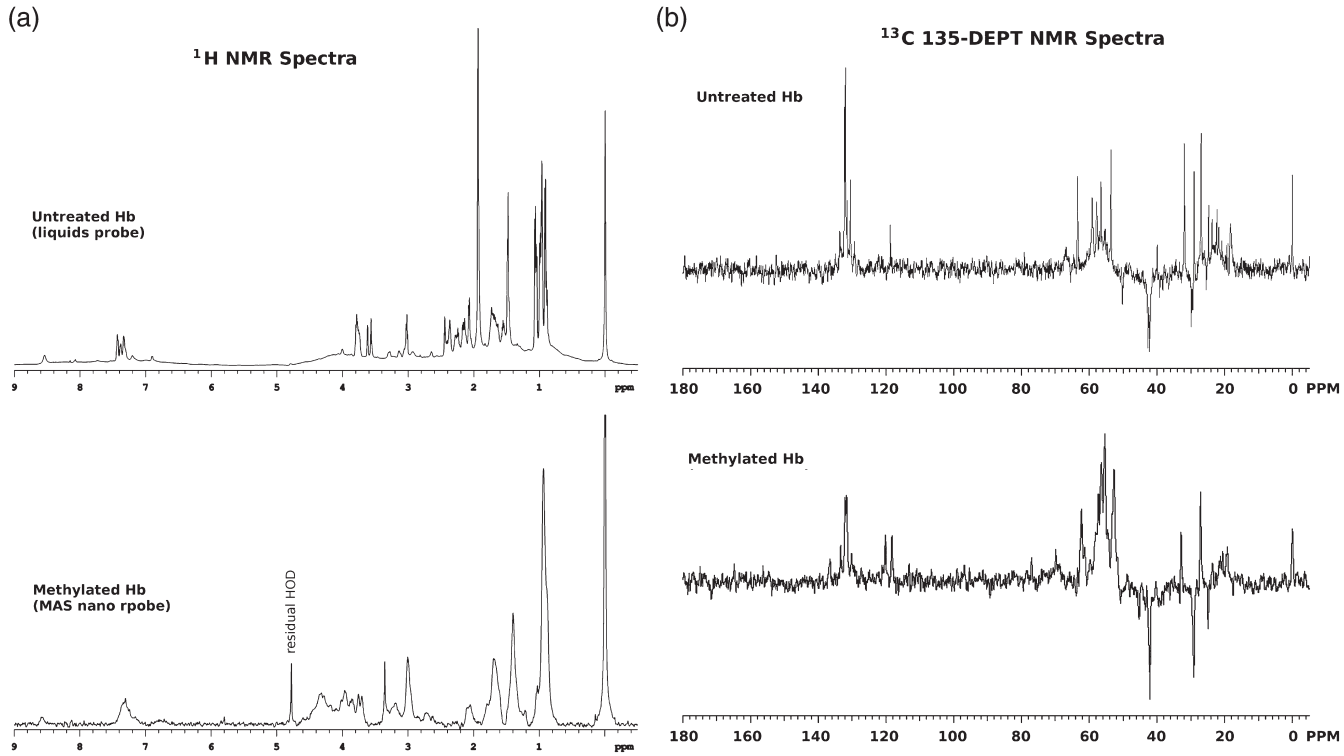

Figure 4. (a) ^{1}H NMR and (b) ^{13}C 135-DEPT NMR spectra of untreated Hb and methylated Hb.

Circular dichroism

Circular dichroism (CD) was used to monitor any changes in the protein secondary structure contents. The formula below was used to convert the results obtained (ellipticity) into mean residue ellipticity (MRE), deg cm^2 dmol^{-1} or deg M^{-1}cm^{-1}:

$$MRE = \frac{\theta_{obs}}{cnlx10} \quad (4)$$

Here, Θ*obs* is the observed ellipticity in mdeg at $\lambda = 222$ nm; *c* is the concentration of the sample in molarity; *n* is the number of amino acid residues in bovine Hb; and *l* is the path length of the cell in cm. The CD spectra (Fig. 2) show changes in the protein secondary structure of Hb compared with MeHb. The percentage of α-helix in the pure Hb was 61%. The α-helix percentage content decrease to 8% upon methylation. The decrease in the α-helix content is due to the unfolding or the perturbation of the secondary structure of the protein. The percentages of α-helix, β-sheet, and random coil were 61%, 7%, and 32%, respectively, for the Hb using the K2d method.[13] The MeHb was composed of 8% α-helix, 48% β-sheet, and 44% random coil. The structure of Hb is mainly dominated by α-helix, while upon methylation the structure was dominated by β-sheet.

Fourier transform infrared spectroscopy (FTIR)

Successful methylation of Hb will result in the blocking of the carboxylic acid group and converting it to an ester functionality. Figure 3 shows the FTIR spectra for both the Hb and MeHb. Both spectra show peaks at 2959 (C–H stretch), 1657 (C=O stretch), 1389 (O–H bend), 1304 (C–O stretch), and 931 (O–H bend) cm^{-1}. As expected, a closer look at the OH region shows the presence of a stronger and broader OH peak in Hb compared with the MeHb. The change in the amount of OH peak was used to confirm whether methylation was achieved.

NMR analysis

While the ^{1}H spectrum of the unaltered Hb sample is sharp and highly resolved, the ^{1}H spectrum of the methylated sample, being slurry-like, remains somewhat broadened even under MAS conditions (Fig. 4(A)). Nevertheless, it is obvious that there are many significant differences between the spectra of Hb and MeHb. While the majority of these differences are difficult to structurally identify it is clear that the ^{1}H spectrum of the MeHb sample has increased intensity between 3.5 and 4.6 ppm. This is probably due to higher concentrations of methoxy groups that are typically found in this spectral region. On the other hand, the non-methylated Hb sample has a tall, sharp peak at 1.93 ppm in the ^{1}H spectrum which seems to vanish in the spectrum of MeHb. The identity of this peak is uncertain, and it may be that it has simply broadened and shifted in the spectrum of the methylated sample.

The solution-state ^{13}C spectra of the two samples (not shown for brevity) are difficult to compare because the slurry-like conditions of the MeHb sample resulted in extremely fast relaxation of the magnetization, producing a low quality spectrum with unreliable signal intensity.

The ^{13}C 135-DEPT spectra (Fig. 4(B)) provided not only better quality data, but also helpful insight into the ^{13}C spectra. This experiment allows one to determine the multiplicity of carbon atoms coupled to attached hydrogens: carbons with an odd number of attached hydrogens (–CH- and –CH_3) are phased positive, carbons with an even number of attached hydrogens (–CH_2-) are phased negative. In overlapping regions of both even and odd numbers of attached hydrogens, the signals tend to cancel. Since quaternary carbons are not detected the analysis is simplified. Alkyl methyl carbons appear below about 30 ppm, whereas –CH_2- carbons occur between ~20 and 40 ppm, and signal cancellation occurs between ~20 and 30 ppm. For example, the greater linewidths of the methylated sample cause the negative peak at

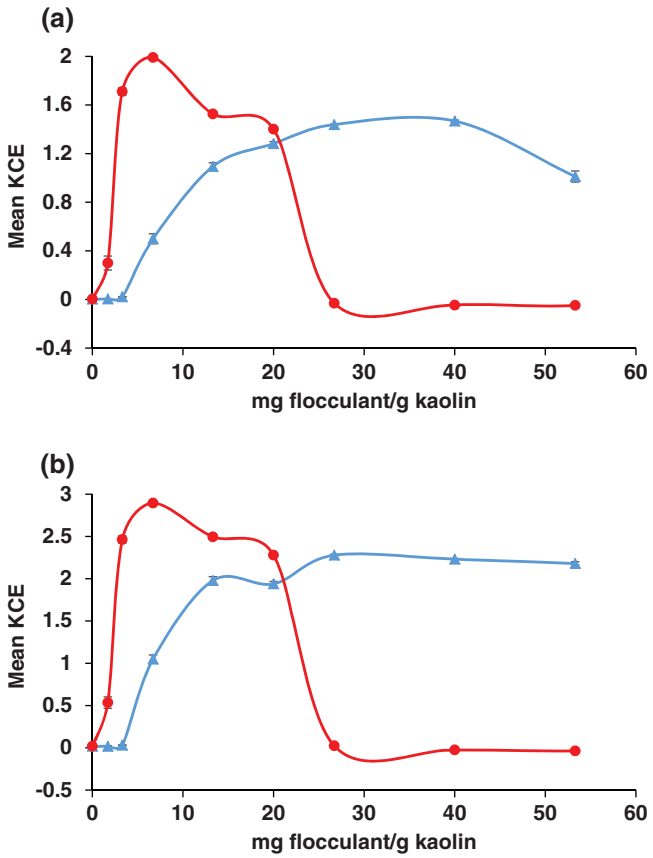

Figure 5. Effect of concentration on flocculation by Hb (▲) and MeHb (●) at (a) 1 h settling time and (b) 6 h settling time. The concentration of Polygloss kaolin in the suspension was 3 g L^{-1} at pH 5.5. Data points are the mean ± SE, n = 4. Error bars are present for all points, but in most cases are too small to visualize.

30 ppm to cancel the intensity of the positive peak at 29 ppm, whereas both peaks can be observed in the sharper untreated sample. With that one exception, the region below ~45 ppm appears to be nearly the same for both samples. Conversely, the methoxy region (~50–60 ppm) is more intense in the methylated sample, indicating an increase in the percentage of this functional group.

Flocculation efficacy

Flocculation studies were carried out using Polygloss 90 kaolin (a clay with the chemical formula $Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$). The kaolin has a surface area of 22.0 m^2 g^{-1} and is well known to exhibit a negative charge. The point of zero charge of kaolin has previously been determined to be in the range of 2.7 to 3.2,[14,15] and thus kaolin should have a net negative surface charge over the entire pH range used in this study. The results of 1 and 6 h flocculation at different flocculant concentrations are shown in Fig. 5(a) and 5(b), respectively. Flocculant concentrations were varied from 0 to 55 mg flocculant per g kaolin while the kaolin suspension was held constant at 3 g L^{-1} and pH 5.5. As shown in Fig. 5(a), the kaolin clarification effectiveness (KCE) increases from ~0 to ~1.47 as the concentration was increased from 0 to 40 mg g^{-1} kaolin in the case of Hb. Above 40 mg g^{-1} kaolin, the flocculation activity starts to decrease as is evident in the decrease in KCE at approximately 53 mg g^{-1} kaolin. However, in the case of MeHb, its KCE increased from ~0 to 2 as its concentration was raised from 0 to ~6.6 mg g^{-1} kaolin. Above 20 mg g^{-1} kaolin, a decrease in flocculating activity is seen for the MeHb. Optimal clarification of kaolin was obtained within a concentration range of 0 to 20 mg g^{-1} kaolin. When higher than optimal doses of flocculant (27 to 53 mg g^{-1} kaolin) are used, the clarification effectiveness drops sharply. This may be due to the re-dispersion of kaolin in the suspension. It is not uncommon for flocculant performance to diminish when used at an excessively high dose.[12] It is clear that the highest KCE at 1 h flocculation was achieved with MeHb at the concentration of ~6.6 mg g^{-1} kaolin.

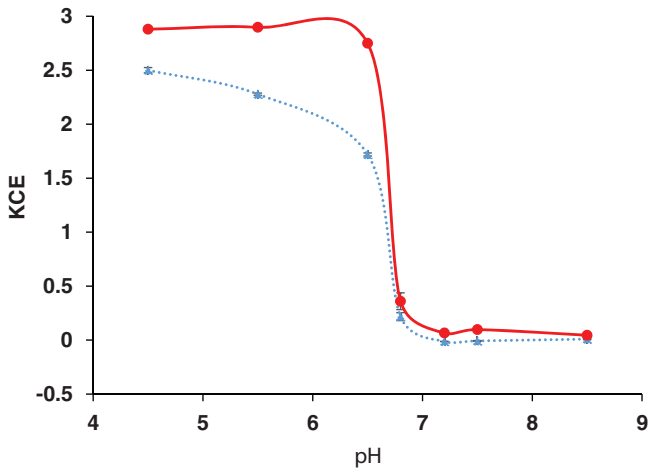

Figure 6. Effect of pH on flocculation by Hb (▲) and MeHb (●) at 6 h settling time. The concentration of Polygloss kaolin in the suspension was 3 g L^{-1}. The dose of flocculant used for the original Hb and MeHb samples were 27 and 7 mg flocculant g^{-1} kaolin, respectively. Data points are the mean ± SE, n = 4. Error bars are present for all points, but in most cases are too small to visualize.

At 6 h flocculation time (Fig. 5(b)), the KCE for Hb remains fairly constant from 13 to 53 mg g^{-1} kaolin, with the highest KCE achieved when the concentration is 27 mg g^{-1}. In the case of MeHb, there is a sharp drop in KCE above 13 mg g^{-1} kaolin. The MeHb KCE values were ≥ 2.3 from 3.3 to 20 mg g^{-1} kaolin, with the highest value obtained at a concentration of 3.3 mg g^{-1} kaolin. It is interesting to note that, at pH 5.5, the amount of hemoglobin needed to achieve approximately the same clarification effectiveness was four times higher in the Hb compared with MeHb. These differences in KCE results between MeHb and Hb are quite large since KCE is a logarithmic scale. For example, a KCE of 3 represents a 1000-fold decrease in turbidity while a KCE of 2 shows that the decrease in turbidity was 100-fold.

The effect of pH on flocculant performance is shown in Fig. 6. The isoelectric point of Hb and methylated Hb determined using Zetasizer Nano were found to be 6 and 8, respectively, (not shown for brevity). As expected, the esterification of carboxyl groups should have the effect of raising the pI. It is well known that lower pH values results in net positive charge on proteins. At lower pH values (that is pH less than the pI of the flocculants), both Hb and MeHb are positively charged while the kaolin is negatively charged. Therefore, when the kaolin suspension is mixed with Hb or MeHb, there is enhanced flocculation as a result of the electrostatic attraction between the positively charged flocculant and the negatively charged kaolin. The flocculants bind to the kaolin, forming flocs, resulting in significant flocculation at lower pH values.

At pH above 6.5 in the case of Hb, the flocculation activity decreases sharply. The pI of Hb is 6. At pH above the pI, Hb acquires a net negative charge. That is, at higher pH values (pH greater than the pI of the flocculant), both the kaolin and flocculant are negatively charged. By methylating the carboxylic acid groups on the protein, the number of negatively charged spots on the protein is reduced. This subsequently diminishes the repulsion between MeHb and the negative charges on the kaolin surface. It is therefore not surprising that the KCE for Hb starts to decrease even at pH 6.5 while that for the MeHb remains approximately constant (Fig. 6). The percentage of original turbidity removed was 37% for Hb while 60% of the original turbidity was removed for MeHb at near-neutral pH (pH = 6.8). Hb actually increased the turbidity of the suspension, above its initial turbidity at all pH values above 6.8; this negative effect was never observed for MeHb. Other authors have shown that methylated egg albumin and soy protein can effectively flocculate diatomite particles over a broad pH range from 3 to 10.[7,8] Nonetheless, it is clear that MeHb is a better flocculant at higher pH than Hb because of the increase in the net positive charge resulting from the blocking of the carboxylic acid groups during methylation.

We point out that it is very hard to make meaningful general comparisons with commercial polymeric flocculants such as polyacrylamide (PAM). Even within a single supplier of PAM flocculants, many varieties are available, and the manufacturer treats chemical characteristics of each preparation as proprietary information. In any particular practical or experimental situation, different varieties of PAM will perform differently.

CONCLUSIONS

Bovine Hb, an inexpensive bioflocculant, has been covalently modified to improve its flocculation activity. MeHb was characterized using different analytical techniques to monitor and confirm the intended change. The flocculant performances of Hb and MeHb were tested with kaolin suspensions at different pH values, settling times, and flocculant doses. At higher pH values (>5.5), MeHb showed better flocculating activity than Hb, making MeHb a more useful industrial flocculant than Hb. Another advantage of MeHb over Hb is that, under some conditions, MeHb clarified suspensions of kaolin at one-quarter the dose of that required by Hb.

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